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THE RESPONSE OF THE TRACHEAL EPITHELIUM TO CONCOMITANT CIS-DIAMMINEDICHLOROPLATINUM (II) AND RADIATION. AN ELECTRON MICROSCOPIC STUDY IN RABBITS

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Abstract

The ciliated epithelium of the rabbit trachea was irradiated with daily fractions of 2 Gy up to an accumulated dose of 20 Gy (total dose: 2, 6, 10, 16, or 20 Gy). Fifteen to forty-five minutes before the start of each irradiation 0.3 mg Cis-diamminedichloroplatinum (cis-DDP) was given by intraperitoneal injection to each rabbit. Examinations were carried out 1-10 days after each fractionation schedule, when specimens were taken for morphological investigations.

Scanning electron microscope (SEM) examination showed a gradual development of ciliary damage, from blebs on the cilia to swollen tips, broken and bent cilia and finally an epithelial injury with areas free from cilia, and a surface covered with microvilli-like structures. SEM also showed cell loss, and remnants of dead cells on the surface together with detritus. By transmission electron microscopy (TEM), ciliary damage, cell death and cell loss of the ciliated cell layer, as well as exfoliation of portions of goblet-like cells on the surface, could be confirmed. Scoring of SEM and TEM micrographs showed that for the tracheal part treated with cis-DDP and radiation, the maximal damage was expressed in the dose group 10 Gy, and above this no further increase in the average reaction occurred. For the part of the trachea only exposed to cis-DDP, the damage increased with the dose. The difference observed speaks for an accelerated proliferation exerted by the radiation.

Key Words: Trachea, epithelium, cilia, scanning electron microscopy, transmission electron microscopy, cis-DDP, radiation.

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Introduction

To obtain successful results in oncology, when treatment is at all possible, requires *inter alia* a high therapeutic ratio (the response of the tumor to the treatment as related to the effect on the normal tissue). If radiation is used together with cytostatic drugs, the dose of the drug (single or repeated) and the timing and sequence between drug and radiation are parameters which have to be taken into consideration.

Cis-diamminedichloroplatinum (cis-DDP) has given promising results together with ionizing radiation. The drug has a documented cytotoxic effect against e.g., testicular and squamous cell carcinoma (Creagan et al., 1981, Coughlin and Richmond, 1985). Moreover, it possesses radio-sensitizing properties (Alvarez et al., 1978; Dionet and Verrelle, 1984), and in combination with radiation it seems to potentiate the radiation effect particularly on malignant tissue as compared to the normal. However, the exact dose and optimal timing in order to obtain the maximal therapeutic ratio is not known. Therefore, an investigation was initiated in order to study the effect of cis-DDP alone or in combination with fractionated radiation, where the drug was given as a high single dose or as repeated daily administration (Albertsson et al., 1986; Albertsson et al., 1987a, b; Albertsson and Hakansson, 1988). The tissues investigated were the mucosa of the trachea and the esophagus. In the current paper cis-DDP has been given daily concomitant with fractionated radiation (2 Gy/F) from a total dose of 2 Gy up to 20 Gy.

Investigations were performed with light microscopy (LM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) 1-10 days after completion of radiation. Damage, repair and repopulation were studied.

Materials and Methods

Animals

Sixty full grown rabbits weighing between 1.9 - 2.4 kg were selected for the study. Fifty received a combination of cis-DDP and radiation according to the

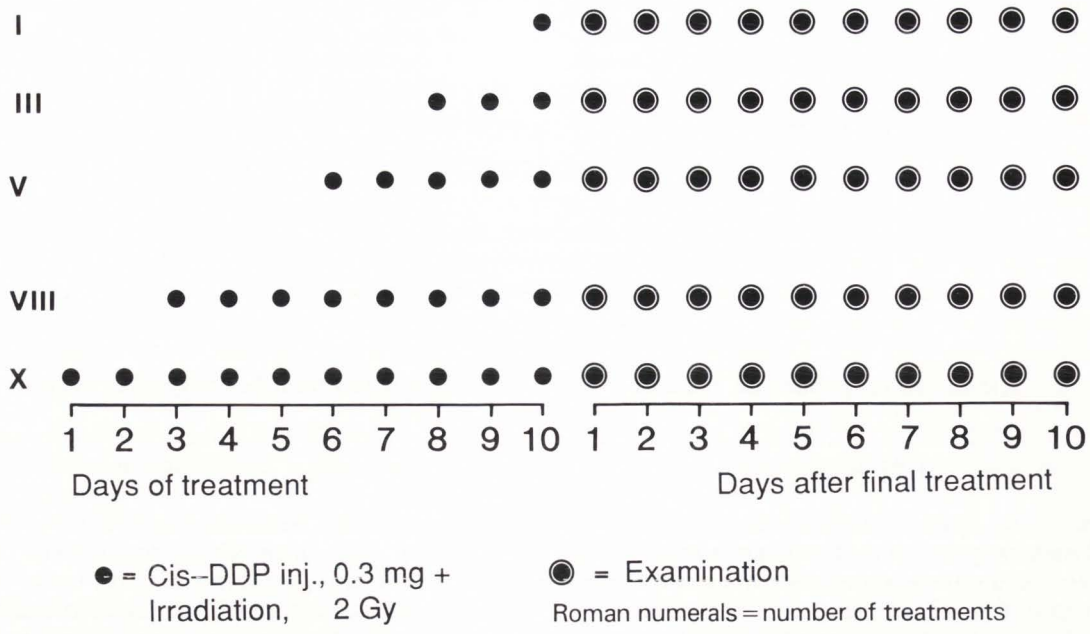


Figure 1. Treatment schedule for combined cis-DDP and irradiation. The drug was given at a dose of 0.3 mg, 15-30 minutes before each irradiation. Total dose ranged from 0.3 mg cis-DDP/2 Gy to 3.0 mg cis-DDP/20 Gy. After completion of treatment, experiments were made daily from day 1 to day 10.

schedule presented in Fig. 1. Ten animals acted as control animals.

Drug

Cis-diamminedichloroplatinum (II) (cis-DDP, cis-platinum), (Platinol®, Bristol Myers Company), was dissolved in isotone saline at a concentration of 0.5 mg/ml.

Radiation

Radiation was delivered by a Siemens X-ray machine operating at 160 kV X-ray, filtered by 4 mm Al, at a focus - skin distance of 50 cm, giving an absorbed dose of 2 Gy to 2 cm of the trachea just beneath the larynx. Fifteen mm beyond the caudal part of the irradiated area, the absorbed dose was 0.05 Gy. The absorbed dose in the trachea was controlled by thermoluminescent dosimeters. The spatial distribution between the irradiated area and control area was 40 mm.

Experiments

Each rabbit was anaesthetized by intraperitoneal injection of pentobarbital (40 mg per kg body weight) before the administration of irradiation.

The rabbits were at first exposed to fractionated irradiation (2 Gy/F), with a total cumulative dose ranging from 2 -20 Gy. Fifteen to forty-five minutes before each irradiation, each animal was given 0.3 mg cis-DDP intraperitoneally according to the schedule in Fig. 1. The rabbits were then laid on their backs and the upper part of each trachea (20 mm) was irradiated. The animals were treated in groups of ten. After completion of radiation, one animal was removed from the group on each of the ten consecutive days. The animal was sacrificed by a blow on the skull, in order to avoid pharmacological side effects. The trachea was dissected out in its entire length (7-8 cm). Samples for SEM, TEM and LM were taken from the upper part of the trachea (irradiated area: T1) and the lower part of the trachea (control area: T2). Control investigations were also performed in the same way on untreated animals.

Preparation for SEM and TEM

The preparation methods have been described in detail in earlier publications in this journal, e.g., see Albertsson and Hakansson (1988).

Statistical Analysis

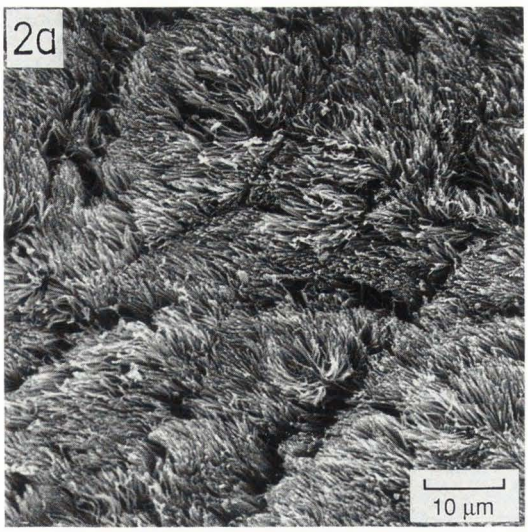
This was done with regression analysis.

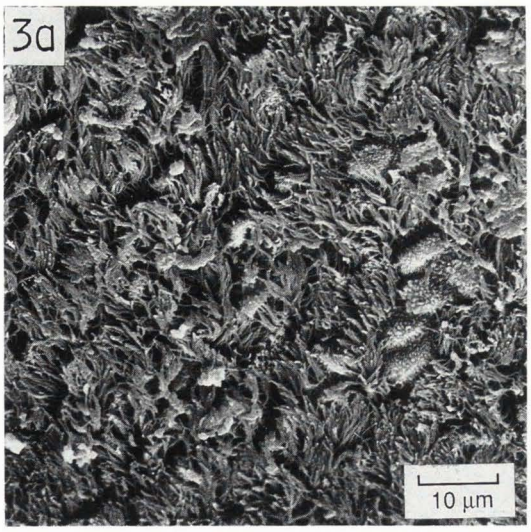
Results

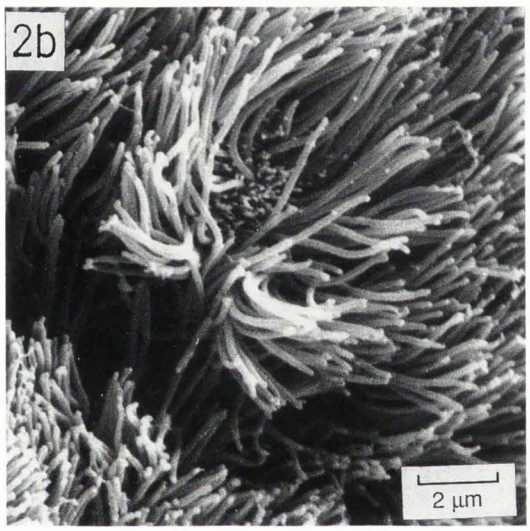
Scanning Electron Microscopy

Untreated control animals. Ultrastructurally, any difference between the upper part of the trachea (T 1) and the lower part (T2) could not be seen. The scanning electron micrographs of the normal ciliary carpet show smooth and regularly arranged cilia with a minimum of blebs. The cilia have a tonicity with a marked bending on the proximal part and with the tips orientated in one direction. Occasionally small whirls are seen, indicating that the cilia are not completely synchronized in their activity. Small openings on the surface indicate cells eventually leaving the surface in a phase of extrusion (Figs. 2a, b).

Treated animals. In evaluating the effects on the morphology of the ciliary carpet after treatment, the results are described as an average reaction (score) on an







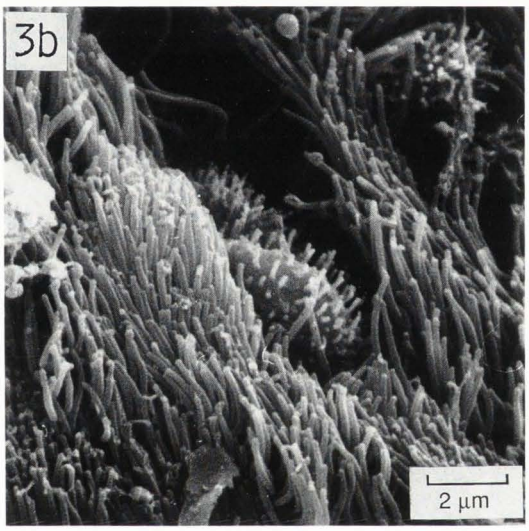


Figure 2 (SEM). Score 0. **(a)** Normal ciliary epithelium. Regularly arranged cilia with a minimum of blebs. **(b)** Enlargement of Figure 2a.

Figure 3 (SEM). Score 1. **(a)** An increased amount of blebs (arrows) and a few Q-cells (arrow). **(b)** Enlargement of Figure 3a.

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arbitrary scale. Scoring of the SEM-surface was made on a defined area (17 x 11 cm at an enlargement of 1000x and 5000x). The areas were chosen at random and at least three different areas were examined from each specimen. In the scale used, 0 shows the normal morphology and 3 the greatest injury. Attention was paid to the cilia, their tonicity and orientation, and also to the epithelial surface as a whole. As described earlier (Albertsson et al., 1986), the ciliary carpet exhibits special formations of damage where the cells have lost the majority of the cilia, but have remnants of microvilli. These areas are designed Q-cells.

When the damage is widespread, most of the carpet is covered by Q-cells. There was no sign of total denudation down to the basal lamina. The score is the average reaction as judged by three independent observers and described in four steps from **0 to 3**:

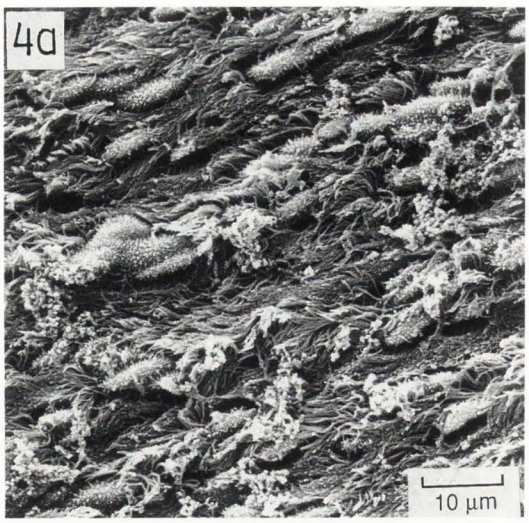
0 = normal ciliary carpet. (Figs. 2a, b).

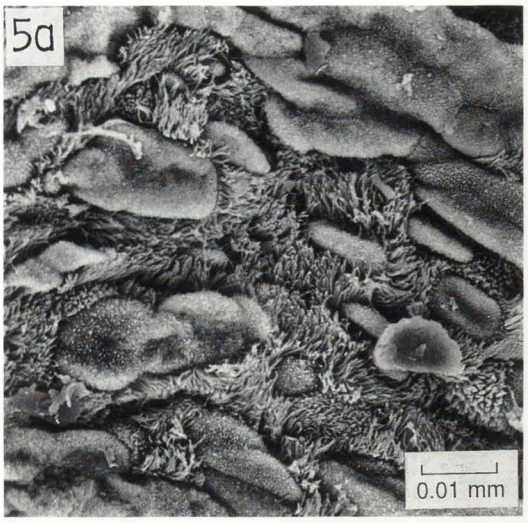
1 = increased amount of blebs, a few Q-cells. (Figs. 3a, b).

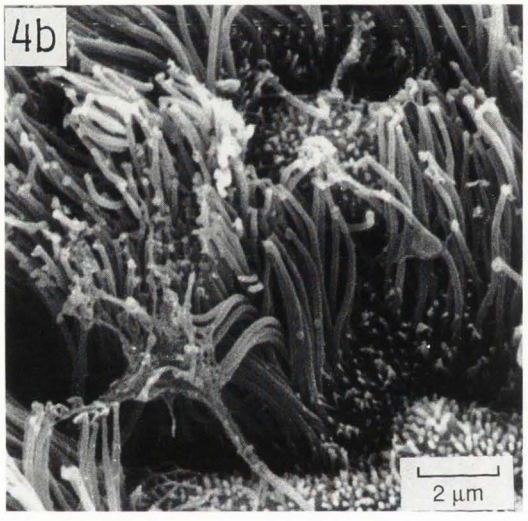
2 = swollen or broken tips of the cilia, many Q-cells. (Figs. 4a, b).

3 = disordered and disintegrated cilia, most of the surface is covered by Q-cells. (Figs. 5a, b).

T1 (area exposed to radiation and Cis-DDP). After treatment, the surface of the mucosa seemed to follow a distinct pattern of degradation in relation to dose. A regression analysis showed the effect of damage







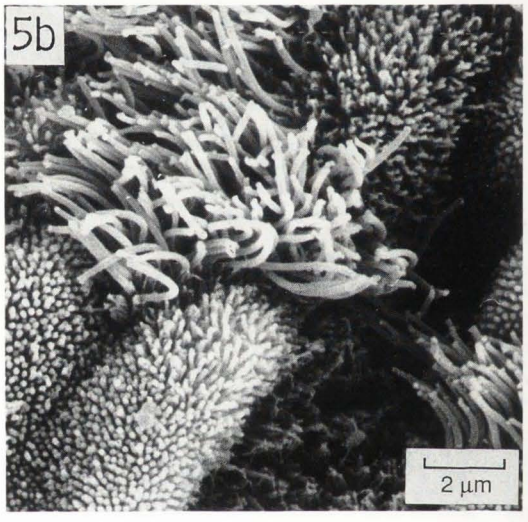


Figure 4 (SEM). Score 2. **(a)** Swollen or broken tips of the cilia (arrows) and many Q-cells. **(b)** Enlargement of Figure 4a.

Figure 5 (SEM). Score 3. **(a)** Disordered disintegrated cilia and most of the surface covered with Q-cells. **(b)** Enlargement of Figure 5a.

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in relation to the given dose to be significant ($p = 0.0134$).

The first effect observed was damage to the cilia with a loss of their tonicity. The normal synchronism had vanished. With increasing dose, a number of blebs of varying size and shape could be seen. These were mostly located on the sides of the cilia but could also be seen on the tips (Figs. 3a, b). The ciliary tips became swollen and sometimes ruptured, apparently emitting a substance. With greater intensity the cilia often fractured (Figs. 4a, b). The breaking position could be situated anywhere on the cilium. The scoring of the scanning electron micrographs is presented in Fig 6. The results from the ten days of examination for each dose group are collected at each point since statistical analysis showed no significant effect as regards the time. For the irradiated and cytostatic-treated part of the trachea, the score was 1.2 in the lowest dose range and reached 1.7 when the accumulated dose was raised to 1.5 mg cis-DDP + 10 Gy. The score was maintained around this level up to 3.0 mg cis-DDP + 20 Gy.

T2 (area only exposed to Cis-DDP). The average reactions in the lower part of the trachea, where only administration had taken place, run parallel to the irradiated part in the low dose range. In part T2, the score value increased with the dose and therefore the two curves diverged substantially in the high dose range (Fig. 6). The difference between the two curves is sta-

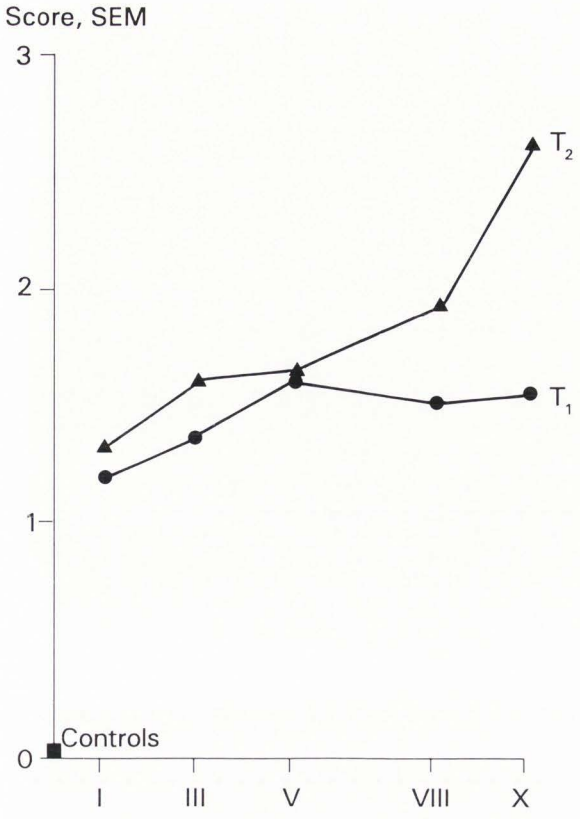

Figure 6. Score mean value expressed for each dose. Each point represents all ten values from each dose group as judged by three people. O = normal, 3 = greatest abnormality [I-X = 1-10 days after treatment: **I** = 0.3 mg cis-DDP + 2 Gy; **III** = 3 x (0.3 mg cis-DDP + 2 Gy); **V** = 5 x (0.3 mg cis-DDP + 2 Gy); **VII** = 8 x (0.3 mg cis-DDP + 2 Gy); **X** = 10 x (0.3 mg cis-DDP + 2 Gy)].

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tistically significant ($p = 0.0002$). At an accumulated dose of 2.4 mg cis-DDP, the damage was a reduction in the number of the cilia on each cell. At 3.0 mg, the epithelium was deprived of cilia and large areas showed Q-cells covered only by microvilli (Figs. 5a, b). However, scoring of the scanning electron micrographs takes only the surface into consideration and for the effects on underlying epithelial cells, evaluation from TEM-micrographs is necessary. Therefore, the scoring of TEM-micrographs was performed with an estimation of the effects on the ciliary cell layers.

Transmission Electron Microscopy

Untreated control animals. The ciliary epithelium consists of columnar ciliated cells with a central nucleus and mitochondria, in most cases located just beneath the anchoring of the cilia into the cells (basal bodies). Normal endoplasmatic reticulum, free ribosomes and well defined Golgi apparatus are seen. Goblet cells of the usual appearance are interspaced between the cells with some of them emptying their content into the lumen. Dark goblet-like cells appear sparsely where the

Figure 7 (TEM). Score 0. Montage illustrating normal epithelium.

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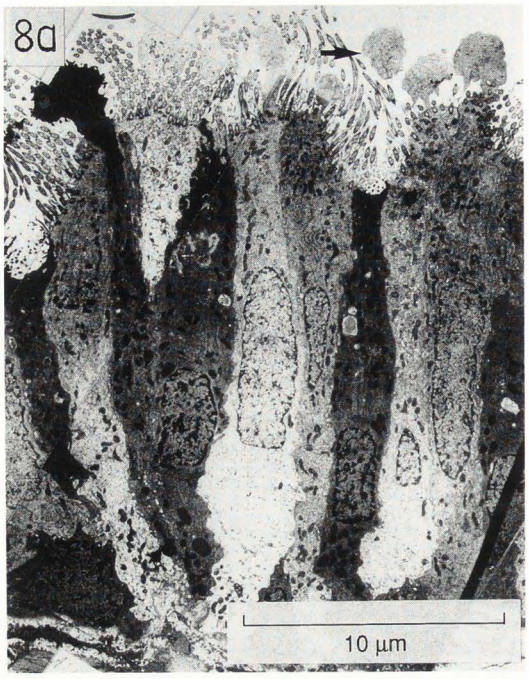
apically situated mitochondria are changed and vacuoles appear. In addition, endocrine cells are seen with a reticular network in the cytoplasm (cisternae) linked to vesicles of different sizes. These cells are often anchored at the base, and from there usually reaches up to the luminal layer. The intermediate cells have a central nucleus and cell-organelles are clearly visible. They are probably on their way to the apex in order to renew the ciliary carpet. At the bottom (basal lamina) are small pyramidal cells intercalated, the so-called basal cells, with a nucleus occupying the major part of the cell and a sparsity of cell organelles (Fig. 7). Hemidesmosomes signify their attachment to the basal lamina.

Treated animals. As far as evaluation of the TEM-micrographs is concerned, a score system was performed from pictures enlarged 7500 x, ranging from 0 - 3, where 0 was judged as normal and 3 the greatest amount of injury:

0 = Normal epithelium. (Fig. 7).

1 = Basal cells intact. Goblet-like cells which often extended from the basal lamina and sometimes protruded on the apical cell surface. Within the cells, granula containing heterogenous material of varying size and shape. Often, the ciliated cells had swollen mitochondria. Cytoplasmic evaginations containing amorphous material were seen to protrude from the apical part of some ciliated cells, surrounded by a double membrane. Reduced number of cilia on the surface, often with blebs and broken tips. (Figs. 8a, b).

2 = The basal cells showed normal ultrastructure although they were reduced in number. Cell death and



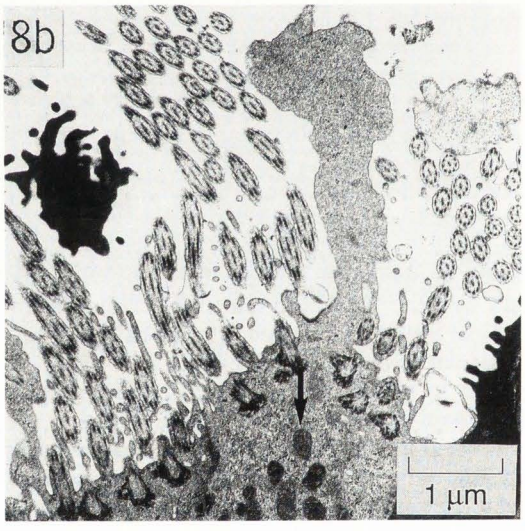
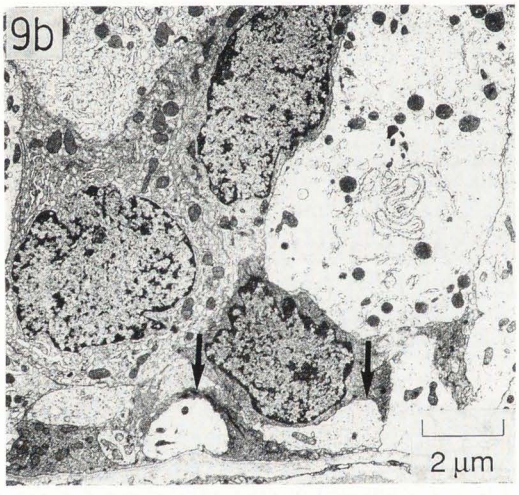


Figure 8 (TEM). Score 1. **(a)** Montage with cytoplasmic evaginations or protrusions (arrow). **(b)** Enlargement of Figure 8a showing swollen mitochondria (arrows).



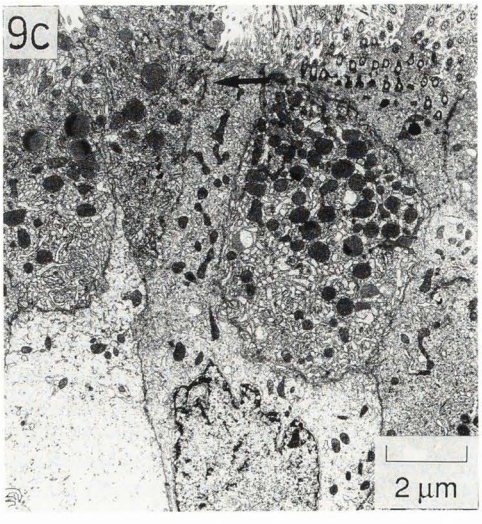
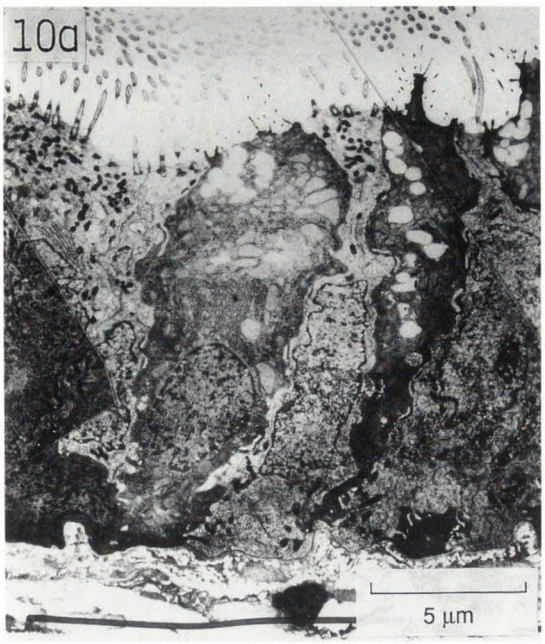


Figure 9 (TEM). Score 2. **(a)** Montage with cell death and cell destruction. **(b)** Basal cell layer and a reduced amount of basal cells (arrows). **(c)** Apical cell layer and dead cells in the phase of extrusion (arrows).



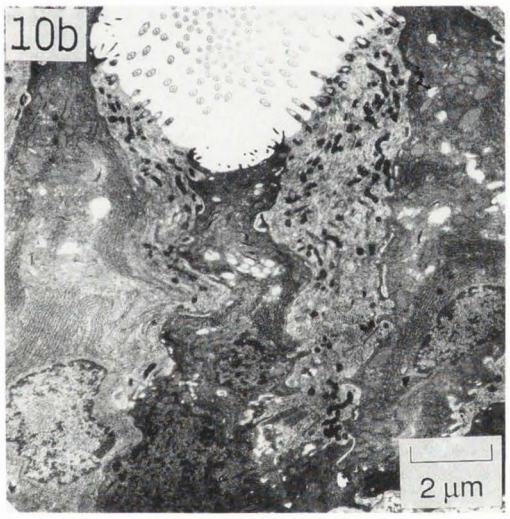


Figure 10 (TEM). Score 3. **(a)** Montage with extensive cell death and cell destruction. **(b)** Enlargement of Figure 10a; the number of cilia is markedly reduced.

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cell destruction often affecting the apical ciliated cell layer with exfoliation of dead cells on the surface. Reduced amount of organelles in the ciliated cells with lysis of mitochondria and, in the nucleus, an increase in the number of perichromatin granules. The cilia on the apical cell surface damaged and reduced in number. (Figs. 9a, b, c).

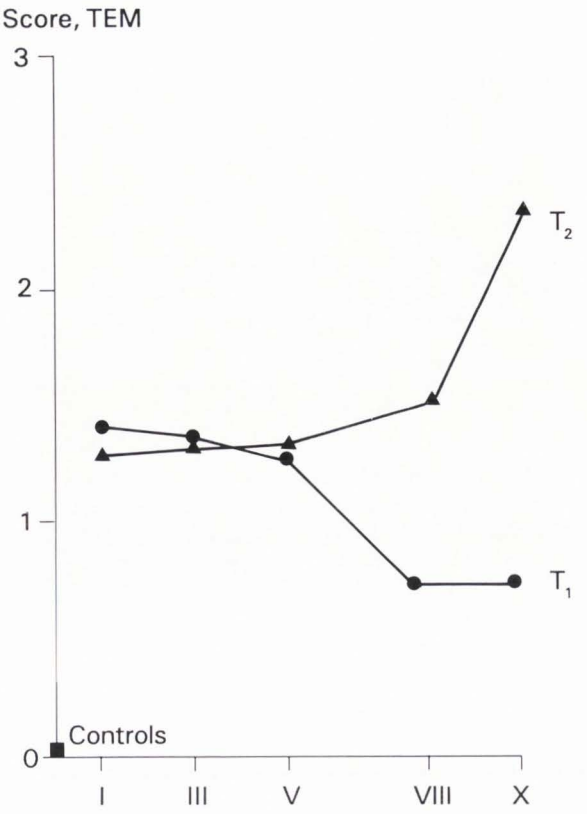

Figure 11. Score mean value expressed for each dose. Each point represents all ten values from each dose group as judged by three people. 0 = normal, 3 = greatest abnormality [I-X = 1-10 days after treatment: **I** = 0.3 mg cis-DDP + 2 Gy; **III** = 3 x (0.3 mg cis-DDP + 2 Gy); **V** = 5 x (0.3 mg cis-DDP + 2 Gy); **VII** = 8 x (0.3 mg cis-DDP + 2 Gy); **X** = 10 x (0.3 mg cis-DDP + 2 Gy)].

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3 = Extensive cell death and cell destruction. Remnants of cilia on the apical surface. Great reduction of the basal cells. (Figs. 10a, b)

The score from the transmission electron micrographs (Fig. 11) results in curves with a course of events similar to the curves for the SEM scoring. T1 and T2 run parallel after 2 Gy, 3 x 2 Gy and 5 x 2 Gy together with cis-DDP with damage most pronounced after 5 x 2 Gy + cis-DDP. With increasing dose (8 x 2 Gy + cis-DDP) a clear difference between T1 and T2 is registered, since the value for T1 remains constant, while the score for T2 increases with increasing dose. The difference between T1 and T2 is seen in Figs. 12a, b. T1 has apparently intact basal cells, normal cell organelles and a large number of cilia at the apical surface. In T2 not exposed for radiation but for cis-DDP only, damage to the apical surface is present with a reduced number of damaged cilia (Fig. 12b). Within the cells, lesions of the cell organelles are seen together with vacuolization of the cytoplasm and mitochondria, in some cases even an extensive necrosis is seen. Damage to the nuclei was

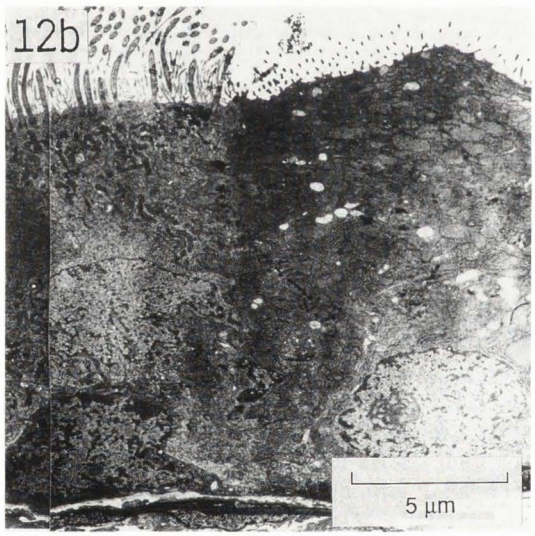

Figure 12 (TEM). **(a).** Montage after treatment with 3 mg cis-DDP + 20 Gy three days after completion of irradiation. T1. **(b).** Montage after treatment with 3 mg cis-DDP three days after completion of treatment.

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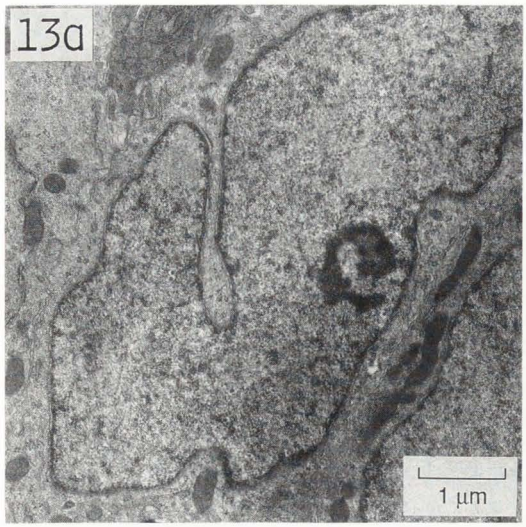
seen as formation of perichromatin and interchromatin granula (Fig. 13), reduction of the number of cell layers and basal cells.



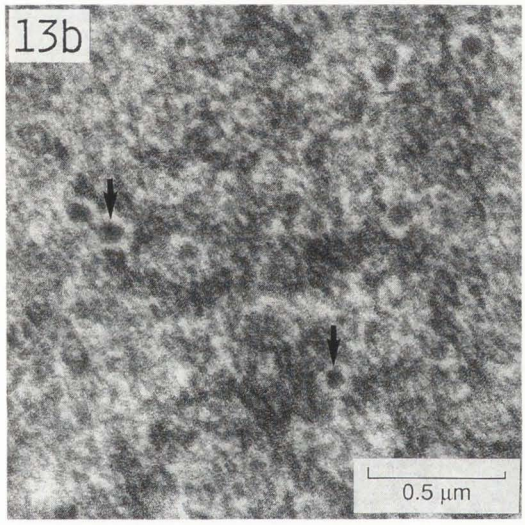


Figure 13 (TEM). **(a).** Nucleus with interchromatin and perichromatin granulae. **(b).** Enlargement of Fig 13a showing interchromatin granulae (arrows).

Discussion

Since a complete cure by cis-DDP has been the exception rather than the rule for most tumors, it is reasonable to suggest that it may be necessary to combine cis-DDP with other modalities for more effective therapy. Cis-DDP has been tested with radiation therapy in different ways. The reason for this is that cis-DDP is expected to act as a radio-sensitizer when combined with radiation. Such a mechanism has been proposed by Richmond and Powers (1976), when they reported the drug as acting as a sensitizer of radiation-induced lethality for hypoxic *B. megaterium*-spores. They suggested the mechanism of action to be on the free radical level. Richmond et al. (1977) reported a maximum

enhancement ratio (ER) of 1.77 for cis-DDP in anoxic bacteria, which suggested that this drug is nearly as effective as a radiation sensitizer as oxygen. It is also reported that the drug inhibits the G1 to S progression and arrests the cells in a radiation-sensitive phase (Szumiel and Nias, 1976). Alvarez et al. (1978) have attributed the enhanced radiation effect to reactions with non-protein sulfhydryl groups and the inhibition of repair processes. A concomitant cis-DDP administration during the course of radiation therapy may lead to increased radiation-induced killing of tumor cells, especially those in the hypoxic cell population, thus improving the therapeutic ratio. In the present study, fractionated radiation with concomitant cis-DDP administration induced cell damage to the ciliated epithelium. The first damage registered on the scanning electron micrographs was a loss of the tonicity of the cilia. With increasing dose, blebs appeared and the cilia broke. At the high dose level, areas covered with only microvilli were seen to an increasing extent. There are many similarities with the results obtained after concomitant cis-DDP and fractionated radiation) but then with the drug administered as a high single dose (5 mg) on day one of a fractionated regime (Albertsson et al., 1986). With the daily administration of the drug, the average reaction of the ciliary damage had a similar course up to the dose-group 10 Gy. With higher doses, the damage in the part treated with a combination of fractionated radiation and cis-DDP tended to level off with a score mean value of 1.4 for 16 Gy and 1.5 for 20 Gy. In the part of the trachea (T2) exposed only to the drug, the score value increased with the dose (Fig. 6). The curves for T1 and T2 diverge substantially in the high dose range. From a number of untreated control animals we know that the ultrastructure is similar for the upper and lower part of the trachea. The difference therefore is likely to be induced by the different treatment modalities. Toxic effects on the ciliated epithelium are not unexpected, since radiomimetics usually act on cells undergoing rapid division like the epithelial basal cells. However, in this investigation, it is worth noting that the part exposed to both cis-DDP and radiation is the one which normalizes, earlier and faster than the part exposed to only cis-DDP (Figs. 6, 11). This may depend on an accelerated proliferation within the tracheal part exposed to the combined treatment, as shown in Figs. 12a, b. It is well known that cells damaged by e.g., ionizing radiation, may result in an accelerated proliferation after a new exposure to radiation. This has been described for the small intestine (Lesher and Bauman, 1969) and for the skin (Denekamp 1982). The dose-dependent aggravation of the ciliary damage noted in the present study as compared to the single dose cis-DDP on day one of the fractionated regime, may depend on the interference with repair (repopulation), since the drug is present during a longer time period in the current experiments. Cis-DDP is known to have an effect on the repair process, both of sublethal radiation damage (SLDR), and of potentially lethal radiation damage (PLDR), (Douple and Richmond, 1979; Dritschilo et al., 1979; Luk et al., 1979). A possible explanation for these results could be the accumulation of the drug when it is given in the form of daily repeated injections. It is known that cis-DDP, once it enters the blood, is taken up very rapidly by tissues (Mattox et al., 1983). At the same time, however, in a slower process cis-DDP as well as its products of hydrolysis, the monoaquo- and diaquo-species, are bound to proteins in the blood and in the tissues (Litterst et al., 1976). It appears that when cis-DDP is bound to macromolecules, proteins, or DNA, it forms a very stable complex which is metabolized very slow, if at all. Therefore, further investigations are planned in which the concentrations of plasma-platinum and tissue-platinum are to be measured simultaneously. Clinical investigations with different therapeutic regimes have been performed on a limited number of patients (Golding and Van Zanten, 1983; Coughlin et al., 1985), and from these no firm conclusions can be made regarding the optimal timing and doses of cis-DDP in order to obtain the maximal therapeutic ratio. However, from these reports, more side-effects have been recorded e.g., pneumonitis with daily administration of the drug as compared to intermittent large injections every third week. Thus, with this way of administering cis-DDP, the ciliary damage is somewhat greater in extent and protracted in time, compared to cis-DDP as a single dose, and the repair process also seems to be delayed (Figs. 12a, b).

The current experiments are therefore to be regarded as preliminary findings and a much longer period of examination is required to obtain a clear picture of the total process of repair.

Conclusion

The combination of cis-DDP and radiation induces damage to the ciliated epithelium. The damage is observed a few days after treatment and shows a gradual development from blebs on the cilia to swollen ciliary tips and broken cilia. Finally, the cilia are lost and large areas of the surface are covered with microvilli-like structures. The maximal damage is expressed in the dose group 10 Gy. However, with further treatment the tracheal part exposed to cis-DDP and radiation normalizes, whilst in the part of the trachea only exposed to cis-DDP the damage increases with the drug dose. The difference may depend on an accelerated proliferation in the part of the trachea that is exposed to a combined treatment.

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Discussion with Reviewers

G.M. Roomans: You have taken tissues at different time points after the end of treatment, but you pool the data. Could you provide information on the time sequence of changes? Were there any signs of normalization towards the end of the 10-day period following the end of treatment?

Authors: We add the statistical analysis of the pooled data. A time sequence is best shown (Table 1 and Figures 14a, b) in the changes of the epithelial height (objective, measured data). We are of the opinion that the use of one animal/ day gives better information on the course of events than e.g., 3 rabbits on day 1, 3 rabbits on day 5, 3 rabbits on day 10, which is a common way to present data.

G.M. Roomans: You state that the cilia in Fig. 4 "emit a liquid substance". Can this really be concluded from a micrograph of fixed tissue?

Authors: No, but we draw this conclusion on the basis of the transmission electron micrographs which show the extrusion of the cytoplasm and we regard this as "a liquid substance".

J. Reitan: Your observation period is 1-10 days after end of treatment. If you instead normalize to start of treatment, which is also the start of cell kinetic perturbations, the mean observation time will be 5 days for the lowest dose and 10 days for the highest dose group. This makes the comparison of your pooled data rather difficult and interferes with your discussion of reactive proliferation. Please comment.

Authors: Your statement is correct and we have paid attention to the difficulty in judging the reaction in our 1986 paper (Albertsson et al., 1986, text reference). The only way to come around this problem is to present data for each day and each fractionation model. Our intention has been to imitate the clinical situation where

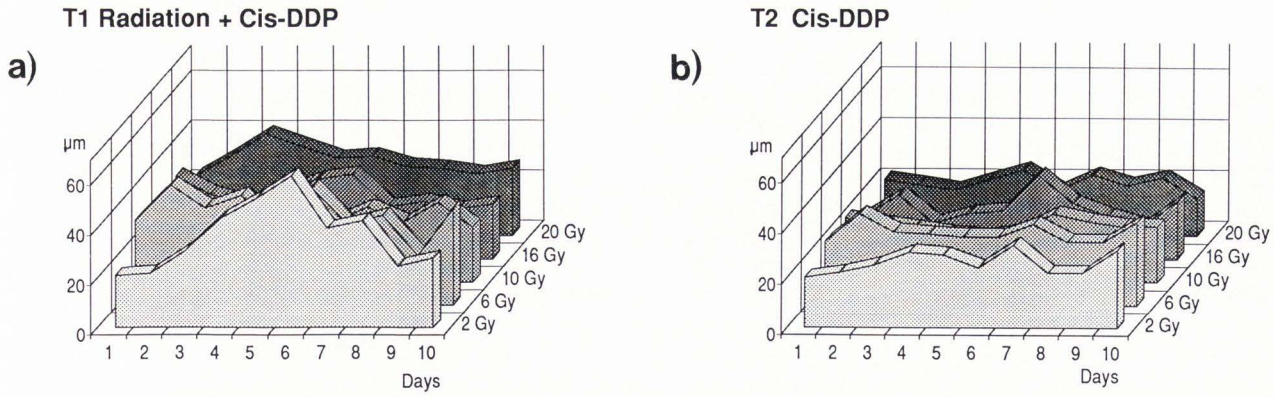

Figure 14. Three-dimensional plot of epithelial height at T1 **(Figure 14a)** and at T2 **(Figure 14b)** as a function of dose and number of days of treatment.

Table 1. Height of the epithelium

Area	Dose Gy	Day 1	2	3	4	5	6	7	8	9	10	mean ± S. D.	normal ± S. D.
T1	2	21	22	31	44	52	61	40	43	25	28		
	6	34	48	39	44	32	47	32	36	20	39		
	10	23	21	20	19	21	24	30	24	32	22	31±9 µm	35±8 µm
	16	33	26	22	27	32	34	21	23	20	22		
	20	24	32	41	36	31	32	28	27	25	27		
T2	2	20	22	25	30	29	24	33	22	22	31		
	6	25	36	29	28	27	27	32	25	25	33		
	10	25	23	21	19	18	20	27	24	22	22	23±5 µm	35±8 µm
	16	17	25	14	18	19	33	22	17	13	23		
	20	22	20	18	22	26	18	24	20	23	15		

we, in the treatment of squamous cell carcinoma of the esophagus, preoperatively treat the patients with concomitant cis-DDP and radiation (24 Gy/12F). The patients are operated directly after completion of irradiation. The side effects are not substantially increased compared to historical controls where only irradiation was given preoperatively. Moreover the local control and survival are better compared to earlier material. Therefore in our animal experimental model system we have been interested in early damage and repair processes after completion of radiation. Since we start our observations already after 2 Gy, early changes may be detectable (cf. Table 1 and Figures 14a, b).

Z. Somosy: What is the possible mechanism(s) of ciliary changes following irradiation and combined treatments?

Authors: The mechanism for ciliary damage after irradiation may depend on membrane damage (H. Dalen: An ultrastructural study of the tracheal epithelium of the guinea pig with special reference to the ciliary structure, J. Anat. 136, 47). Also the edema may contribute to the damage. As far as the combined treatment is concerned, we observe a cytotoxic effect on the ciliary cells.

Z. Somosy: Could the authors give data on changes in the structure of ciliary microtubular doublets and/or basal bodies?

Authors: In the low dose range no structural changes of the microtubules are seen. In the high dose range several of the cilia contain amorphous material and a disorientation of the basal bodies is noted.